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Short Communication

Isolation and characterization of 16 polymorphic microsatellite loci for the Asian green mussel *Perna viridis* (Mollusca, Mytilidae)

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Abstract

The Asian green mussel *Perna viridis* is an abundant and important ecological and economical species across its native range. However, outside its native range, this species has been considered invasive and concerns have been raised worldwide regarding its potential impacts. Despite this, little work has been done to investigate the genetics of native and/or introduced populations of this species. In the present study, we developed 16 new polymorphic microsatellite markers using the Illumina MiSeq Platform. Four to 15 alleles per locus were detected. There was no evidence of linkage disequilibrium between pairs of loci and all loci were in Hardy-Weinberg equilibrium.

Key words: marine pest, biosecurity, genetic markers, *Perna viridis*, population genetics

Introduction

The Asian green mussel *Perna viridis* (Linnaeus, 1758) occurs extensively throughout the Indo-Pacific region from the Persian Gulf, throughout India and South East Asia, to China and Japan (Baker et al. 2007; Siddall 1980). This species' native and introduced range within Asian waters is, however, unclear. *Perna viridis* are fouling organisms that are likely to have increased their range through centuries of shipping in the region (Baker et al. 2007; Hanyu and Sekiguchi 2000; Siddall 1980). They are also an important food source and have been intensively farmed, and translocated for farming, in South East Asia since the 1950's (Ye 1997; Vakily 1989). Intentional (aquaculture) and unintentional (shipping) introductions of *P. viridis* outside of its Asian range have further occurred to numerous Pacific islands (Eldredge 1994), the Caribbean (Agard et al. 1993) and North and South America within the Atlantic Ocean (Benson et al. 2001; Power et al. 2004; Rylander et al. 1996).

The successful establishment of this species outside its native range has had concerning economic and ecological impacts in some areas e.g. south-east coast of the USA (Ingrao et al. 2001; Benson et al. 2001; Gilg et al. 2012). In Australia, *P. viridis* is among the most commonly identified target pest species within the biofouling community of vessels

entering Australian waters (McDonald 2012; Piola and McDonald 2012) and is listed under the National System for Prevention and Management of Marine Pest Incursions (DAFF 2010). In Indonesia, concerns have been raised recently regarding the role of domestic shipping as vectors of introduction and dispersal across native (west) and non-native (east) bioregions (Huhn et al. 2015).

Despite the worldwide distribution, abundance and overall importance of this species, only a few studies have addressed its population genetics, with most occurring within its native range and using a small number of populations and/or markers (Gilg et al. 2012; Lin et al. 2012; Prakoon et al. 2010). Genetic markers can be used to characterise genetic diversity and are therefore able to provide important information needed to identify native and introduced ranges and investigate potential frequency and routes of colonisation and dispersal (Cristescu 2015; Holland 2000). The success of being able to track invasion routes however depends on a balance between sampling effort, type and number of markers used and, ultimately, the species native range genetic structure (Estoup and Guillemaud 2010; Geller et al. 2010; Holland 2000). Fast evolving mitochondrial markers such as COI are commonly used to investigate taxonomy, phylogeography and population genetics of metazoan species (Estoup and Guillemaud 2010). While the maternally transmitted mitochondrial COI marker can be more sensitive to genetic drift, abundant and hypervariable nuclear microsatellite markers tend to give higher population structure resolution (Darling et al. 2008; Holland 2000).

A total of 37 microsatellite markers developed specifically for *P. viridis* are publicly available in the scientific literature (10 by Lin et al. 2007, 19 by Ong et al. 2005, 2008 and 2009, and 8 by Cao et al. 2013). However, these markers were developed using the enrichment technique and only Lin et al. 2007 used a DNA sequencer to obtain allelic data. Upon testing, we found only six of these 37 loci to be consistently scoreable. Therefore, we developed 16 new markers because a greater number of loci are needed to support population and bioinvasion genetic studies on *P. viridis* at native and introduced locations.

Materials and methods

Next-generation sequencing

Genomic DNA (2.6 µg) was isolated from a 25 mg tissue sample of a *P. viridis* using a Fisher Biotec Favorgen FavorPrep Tissue Genomic DNA Extraction Mini Kit. The DNA was sent to the Australian Genomic Research Facility (AGRF, Melbourne) for sequencing using an Illumina MiSeq Platform. Libraries were prepared with the TruSeq DNA Nano using the 550 base pair (bp) insert protocol, which includes shearing and bead size selection at the AGRF. Sequencing was performed at the AGRF with 300 bp paired end reads on the Illumina MiSeq. The data was de-multiplexed as part of the sequencing protocol options. The sequences were assembled into paired reads using PEAR v0.9.7 (Zhang et al. 2014), with a q-value cut-off of 20. These paired reads were scanned for Simple Sequence Repeats (SSRs) and a list of primer sequences and PCR conditions was generated for pure microsatellites using the open source QDD v1.3 (Meglécz et al. 2010) and Primer3 v2.3.3 (Rozen and Skaletsky 2000) software following Gardner et al. (2011).

Primer testing

We selected 39 di-, tri-, tetra-, and penta-base repeat microsatellite loci with a PCR product of 100–400 bp for further development. These loci were trialled for amplification separately in 5 µl reactions containing 10 ng of DNA, 1 × MyTaq reaction buffer (containing 5 mM dNTP and 15 mM $MgCl_2$), 0.5 U MyTaq DNA polymerase (Bioline Reagents), and 0.2 µM of each primer. The following PCR conditions were used: 95 °C for 3 min followed by 30 cycles at 95 °C for 30 s, an optimal annealing temperature (Table 1) for 45 s, and 72 °C for 30 s, and a final elongation step at 72 °C for 5 min. PCR products were visualized on 3 % agarose gels stained with GelRed (Biotium Inc.) alongside a 100 bp molecular weight marker (Axygen Biosciences) and visualised under UV light. Loci which generated a product of the expected size were tested for polymorphism using DNA extracted from eight individual *P. viridis* mussels collected by hand from along the Kaohsiung river (22º37′23.57″N, 120º16′10.02″E), a built up area in Taiwan with considerable boating activity.

Each of the forward primers for polymorphic loci selected for fragment analysis were labelled with a fluorescent tag: FAM (GeneWorks), NED, PET or VIC (Applied Biosystems) and screened for variation using 37 *P. viridis* collected by hand from mussel aquaculture farms in Jakarta Bay (06º04′0″S, 106º43′0″E), Indonesia. PCR products (2.5 µl) were analysed on an ABI 3730 Sequencer, sized using the GeneScan-500 LIZ internal size standard and scored using GENEMARKER software (SoftGenetics).

We used CERVUS (Kalinowski et al. 2007) to calculate the number and range of allele sizes, polymorphic information content, observed and expected heterozygosity and the frequency of null alleles for each locus. To test for deviation from Hardy-

Table 1. Primer sequences, GenBank accession numbers, annealing temperatures, repeat motif, and levels of diversity for 16 microsatellite loci in marine mussel *Perna viridis*. Number of alleles (Na), polymorphic information content (PIC), observed heterozygosity (H_O), expected heterozygosity (H_E), probability value from a test for deviation from Hardy-Weinberg Equilibrium (*P*) and null allele frequency (F). F, N, V, and P indicate dyes FAM, NED, VIC, and PET respectively.

Locus	Primer Sequence (5'-3')	GenBank Acc. no.	Annealing Temp	Repeat Motif	Na	Size Range (bp)	PIC	H_o	H_E	*P*	F (Null)
Pv02[P]	F:ATGGAACATCTCGAGTGCAA R:CGTTTGACTTTCCAACCTTCA	KX463418	53°C	(AAAC)9	8	230-259	0.58	0.61	0.63	0.59	-0.01
Pv05[N]	F:TACTGCATGCTGCTCCTCTG R:AGCAATTAACTCGGAACAGTTTCT	KX463419	53°C	(AATC)7	11	333-372	0.83	0.81	0.86	0.87	0.02
Pv06[N]	F:AATTTAGATCTTCTCAATCGCCC R:AGCATAGCATGTCTGTTGTCTTC	KX463420	60°C	(AATC)9	7	224-248	0.62	0.61	0.67	0.56	0.04
Pv13[F]	F:CTGCGTTAGCACTTGCTTTG R:TTCCCAATTAGTCATCGTTCA	KX463421	53°C	(AAT)15	12	130-168	0.85	0.77	0.88	0.23	0.05
Pv15[V]	F:TCAATAGGGAATGATATGAAGGA R:CAAATCGAACACCAGGATGA	KX463422	53°C	(AAT)14	8	195-220	0.79	0.85	0.83	0.30	-0.02
Pv17[F]	F:TGAAAGATCAAAGGATAGCTTAAAGG R:CATGTGCATGTAAATGACCAAA	KX463423	53°C	(AAT)9	5	156-168	0.61	0.62	0.66	0.05	0.05
Pv18[F]	F:TTTCTTGAAAGCAAACAGTTACG R:TGAGAAACCAAGACGCTGAA	KX463424	53°C	(AAT)15	12	127-164	0.85	0.78	0.88	0.19	0.05
Pv21[V]	F:GCTAGGTTTCATCCTTAATAACATTG R:ATCCATGTCCAATGCACAAA	KX463425	53°C	(AAT)14	5	130-146	0.59	0.69	0.65	0.59	-0.05
Pv22[N]	F:TGACATTATCATGTAGAACATCTCAA R:TCATTCAAACCTGTCTGTGCTT	KX463426	53°C	(AC)12	9	252-268	0.74	0.78	0.78	0.10	-0.02
Pv26[P]	F:AGTCCCTTCTCCTCGCTGAT R:AAAGACAGTTTAGGCGTTCCA	KX463427	60°C	(AC)10	7	189-201	0.70	0.73	0.74	0.96	0.00
Pv30[V]	F:GGCACCAGTAATGCTGTTCTC R:TTTGAAGCATACCAATTACAGTGA	KX463428	53°C	(AC)10	12	192-215	0.85	0.91	0.88	0.66	-0.03
Pv31[P]	F:TGCATATTACTCATTCACCACAAG R:TGTATTTCAGCAAATTGGCATT	KX463429	53°C	(AC)9	4	188-194	0.23	0.22	0.25	0.05	0.05
Pv32[N]	F:GGCCGAGGTACATTTGTGAG R:TCACCAAACTAACATATTCCGAGA	KX463430	53°C	(AC)8	4	127-134	0.46	0.39	0.57	0.07	0.18
Pv33[P]	F:TGTCTCAATACCATGGCGAA R:TGCCTACTTGATACCATTCGAT	KX463431	53°C	(AC)9	6	214-227	0.64	0.70	0.68	0.72	-0.01
Pv34[N]	F:TCAGACTGCACACTGAGTCAAA R:TTGCAAACACATTTCAAGCA	KX463432	53°C	(AG)9	15	145-181	0.90	0.90	0.92	0.40	0.00
Pv37[F]	F:CCACACCTGTACATAGCCTGA R:GAAAGCAGGTTCATTGGGTG	KX463433	60°C	(AC)9	13	176-209	0.76	0.70	0.79	0.38	0.05

Weinberg equilibrium and linkage disequilibrium between pairs of loci, we used the online version of GENEPOP 4.0 (Raymond and Rousset 1995). All pairwise tests were adjusted for multiple tests by false discovery rate (FDR) correction (Benjamini and Yekutieli 2001).

Results and discussion

The sequence run yielded 890,475 quality paired reads with the sequences between 50–590 bp having a peak at approximately 550 bp. There were 14,404 pure microsatellites of >4 repeats for which primers were designed. Thirty four of the 39 targeted loci generated a product of the expected size. Of the 39 loci initially screened, 27 (69%) produced PCR products with clear bands and appeared polymorphic after agarose gel electrophoresis. From these, 16 loci produced genotypes that were consistently scoreable. The number of alleles per locus ranged from four to 15 and the observed and expected heterozygosities ranged between 0.22 to 0.91, and 0.25 to 0.92 respectively (Table 1). All loci were in Hardy-Weinberg equilibrium, and there was no evidence of linkage disequilibrium between any pair of loci.

Studies looking at differences between the enrichment technique and the next-generation sequencing technique (Gardner et al. 2011; Abdelkrim et al. 2009) have found the latter to recover more useable loci, as it targets all microsatellite repeat types (e.g. di-, tri-, tetra- penta- and hexanucleotides). The high number of perfect and polymorphic loci developed in this study, should allow for a higher success when attempting to reproduce its application. Further, they will be useful to future genetic diversity and bioinvasion studies of *P. viridis*, supporting a science-based management approach to the future prevention and management of this species.

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References

Abdelkrim J, Robertson BC, Stanton JAL, Gemmell NJ (2009) Fast, cost effective development of species-specific microsatellite markers by genomic sequencing. *BioTechniques* 46: 185–192, https://doi.org/10.2144/000113084

Agard J, Kishore R, Bayne B (1993) *Perna viridis* (Linnaeus, 1758): first record of the Indo-Pacific green mussel (Mollusca: Bivalvia) in the Caribbean. *Caribbean Marine Studies* 3: 17–22

Baker P, Fajans JS, Arnold WS, Ingrao DA, Marelli DC, Baker SM (2007) Range and dispersal of a tropical marine invader, the Asian green mussel, *Perna viridis*, in subtropical waters of the southeastern United States. *Journal of Shellfish Research* 26: 345–355, https://doi.org/10.2983/0730-8000(2007)26[345:RADOAT]2.0.CO;2

Benjamini Y, Yekutieli D (2001) The control of the false discovery rate in multiple testing under dependency. *The Annals of Statistics* 29: 1165–1188

Benson AJ, Marelli DC, Frischer ME, Danforth JM, Williams JD (2001) Establishment of the green mussel, *Perna viridis* (Linnaeus, 1758) (Mollusca: Mytilidae) on the west coast of Florida. *Journal of Shellfish Research* 20: 21–29

Cao YY, Li ZB, Li QH, Chen XJ, Chen L, Dai G (2013) Characterization of eight novel microsatellite markers in the green-lipped mussel *Perna viridis* (Mytilidae). *Genetics and Molecular Research* 12: 344–347, https://doi.org/10.4238/2013.February.7.4

Cristescu ME (2015) Genetic reconstructions of invasion history. *Molecular Ecology* 24: 2212–2225, https://doi.org/10.1111/mec.13117

DAFF (2010) Australian Marine pest monitoring guidelines. The National System for the Prevention and Management of Marine Pest incursions. Department of Agriculture, Fisheries and Forestry, 57 pp

Darling JA, Bagley MJ, Roman J, Tepolt CK, Geller JB (2008) Genetic patterns across multiple introductions of the globally invasive crab genus *Carcinus*. *Molecular Ecology* 17: 4992–5007, https://doi.org/10.1111/j.1365-294X.2008.03978.x

Eldredge LG (1994) Introductions of commercially significant aquatic organisms to the Pacific Islands. South Pacific Commission Inshore Fisheries Research Project Technical Document 7. New Caledonia: South Pacific Commission, 127 pp

Estoup A, Guillemaud T (2010) Reconstructing routes of invasion using genetic data: why, how and so what? *Molecular Ecology* 19: 4113–4130, https://doi.org/10.1111/j.1365-294X.2010.04773.x

Gardner MG, Fitch AJ, Bertozzi T, Lowe AJ (2011) Rise of the machines – recommendations for ecologists when using next generation sequencing for microsatellite development. *Molecular Ecology Resources* 11: 1093–1101, https://doi.org/10.1111/j.1755-0998.2011.03037.x

Geller JB, Darling JA, Carlton JT (2010) Genetic perspectives on marine biological invasions. *Annual Review of Marine Science* 2: 367–393, https://doi.org/10.1146/annurev.marine.010908.163745

Gilg MR, Johnson EG, Gobin J, Bright BM, Ortolaza AI (2012) Population genetics of introduced and native populations of the green mussel, *Perna viridis*: determining patterns of introduction. *Biological Invasions* 15: 459–472, https://doi.org/10.1007/s10530-012-0301-2

Hanyu K, Sekiguchi H (2000) *Perna* mussels introduced into Ise and Mikawa Bays, Central Japan. *Sessile Organisms* 17: 1–11, https://doi.org/10.4282/sosj.17.1

Holland BS (2000) Genetics of marine bioinvasions. *Hydrobiologia* 420: 63–71, https://doi.org/10.1023/A:1003929519809

Huhn M, Zamane NP, Lenz M (2015) A ferry line facilitates dispersal: Asian green mussels *Perna viridis* (Linnaeus, 1758) detected in eastern Indonesia. *BioInvasions Records* 4: 23–29, https://doi.org/10.3391/bir.2015.4.1.04

Ingrao DA, Mikklesen PM, Hicks DW (2001) Another introduced marine mollusk in the Gulf of Mexico: the Indo-Pacific green mussel, *Perna viridis*, in Tampa Bay, Florida. *Journal of Shellfish Research* 20: 13–19

Kalinowski S, Taper M, Marshall T (2007) Revising how the computer program CERVUS accommodates genotyping error increases success in paternity assignment. *Molecular Ecology* 16: 1006–1099, https://doi.org/10.1111/j.1365-294X.2007.03089.x

Lin G, Feng F, Yue G (2007) Isolation and characterization of polymorphic microsatellites from Asian green mussel (*Perna viridis*). *Molecular Ecology Notes* 7: 1036–1038, https://doi.org/10.1111/j.1471-8286.2007.01765.x

Lin G, Lo LC, Yue GH (2012) Genetic Variations in Populations from Farms and Natural Habitats of Asian Green Mussel, *Perna viridis*, in Singapore Inferred from Nine Microsatellite Markers. *Journal of the World Aquaculture Society* 43: 270–277, https://doi.org/10.1111/j.1749-7345.2012.00558.x

McDonald JI (2012) Detection of the tropical mussel species *Perna viridis* in temperate Western Australia: possible association between spawning and a marine heat pulse. *Aquatic Invasions* 7: 483–490, https://doi.org/10.3391/ai.2012.7.4.005

Meglécz E, Costedoat C, Dubut V, Gilles A, Malausa T, Pech N, Martin JF (2010) QDD: a user-friendly program to select microsatellite markers and design primers from large sequencing projects. *Bioinformatics* 26: 403–404, https://doi.org/10.1093/bioinformatics/btp670

Ong LCC, Tan SG, Yusof K, Yap CK (2005) Isolation of DNA microsatellite markers in the green-lipped mussel, *Perna viridis*. *Pertanika Journal of Tropical Agricultural Science* 28: 79–85

Ong CC, Teh CH, Tan SG, Yusoff K, Yap CK (2008) Eleven novel polymorphic microsatellite DNA markers from the green-lipped mussel *Perna viridis*. *Russian Journal of Genetics* 44: 498–500, https://doi.org/10.1134/S1022795408040170

Ong CC, Yusoff K, Yap C, Tan S (2009) Genetic characterization of *Perna viridis* L. in peninsular Malaysia using microsatellite markers. *Journal of Genetics* 88: 153–163, https://doi.org/10.1007/s12041-009-0023-0

Piola RF, McDonald JI (2012) Marine biosecurity: The importance of awareness, support and cooperation in managing a successful incursion response. *Marine Pollution Bulletin* 64: 1766–1773, https://doi.org/10.1016/j.marpolbul.2012.06.004

Power AJ, Walker RL, Payne K, Hurley D (2004) First occurrence of the nonindigenous green mussel, *Perna viridis* (Linnaeus, 1758) in coastal Georgia, United States. *Journal of Shellfish Research* 23: 741–744

Prakoon W, Tunkijjanukij S, Nguyen TTT, Na-Nakorn U (2010) Spatial and Temporal Genetic Variation of Green Mussel, *Perna viridis* in the Gulf of Thailand and Implication for Aquaculture. *Marine Biotechnology* 12: 506–515, https://doi.org/10.1007/s10126-009-9234-x

Raymond M, Rousset F (1995) GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. *Journal of Heredity* 86: 248–249

Rozen S, Skaletsky HJ (2000) Primer3 on the WWW for general users and for biologist programmers. In: Krawetz S, Misener S (eds), Bioinformatics Methods and Protocols: Methods in Molecular Biology, Humana Press, Totowa, NJ, pp 365–386

Rylander K, Perez J, Gomez JA (1996) Status of the green mussel, *Perna viridis* (Linnaeus, 1978) (Mollusca: Mytilidae), in northeastern Venezuela. *Caribbean Marine Studies* 5: 86–87

Siddall SE (1980) A classification of the genus *Perna* (Mytilidae). *Bulletin of Marine Sciences* 30: 858–870

Vakily JM (1989) The biology and culture of mussels of the genus *Perna*. ICLARM Studies and Reviews 17. International Centre for Living Aquatic Resources Management, Manila, Philippines, 63 pp

Ye W (1997) On experimental transplantation of *Perna viridis* (Linnaeus) along the Guangxi coast. *Studia Marina Sinica/Haiyang Kexue Jikan* 39: 59–63

Zhang J, Kobert K, Flouri TS, Stamatakis A (2014) PEAR: a fast and accurate Illumina Paired-End read merger. *Bioinformatics* 30: 614–620, https://doi.org/10.1093/bioinformatics/btt593